Essential Role of Tumor Necrosis Factor α in Alcohol-Induced Liver Injury in Mice

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Background & Aims: **Tumor necrosis factor (TNF)-α is associated with increased mortality in alcoholics, but its role in early alcohol-induced liver injury is not fully understood. Recently, it was shown that injury induced by the enteral alcohol delivery model of Tsukamoto and French was reduced by antibodies to TNF-α. To obtain clear evidence for or against the hypothesis that TNF-α is involved, we studied TNF receptor 1 (TNF-R1, p55) or 2 (TNF-R2, p75) knockout mice.** ***Methods:*** **Long-term enteral alcohol delivery was modified for male gene–targeted mice lacking TNF-R1 and TNF-R2. Animals were given a high-fat liquid diet continuously with either ethanol or isocaloric maltose-dextrin as a control for 4 weeks.** ***Results:*** **Ethanol elevated serum levels of alanine aminotransferase nearly 3-fold in wild-type and TNF-R2 knockout mice but not in TNF-R1 knockout mice. Likewise, ethanol caused severe liver injury in wild-type mice (pathology score, 5.5 ± 0.6) and TNF-R2 knockout mice (pathology score, 5.0 ± 0.4), but not in TNF-R1 knockout mice (pathology score, 0.8 ± 0.4; $P < 0.001$).** ***Conclusions:*** **Long-term ethanol feeding caused liver injury in wild-type and TNF-R2 knockout mice but not in TNF-R1 knockout mice, providing solid evidence in support of the hypothesis that TNF-α plays an important role in the development of early alcohol-induced liver injury via the TNF-R1 pathway. Moreover, the long-term enteral ethanol feeding technique we described for the first time for knockout mice provides a useful new tool for alcohol research.**

Although the histopathology of early alcoholic liver disease, i.e., steatosis, inflammation, and necrosis, has been well documented,[1] the exact mechanisms of pathogenesis of this devastating disease are still largely unknown. Early ethanol-induced liver injury can be prevented by treatment with gadolinium chloride, a selective Kupffer cell toxicant, indicating that Kupffer cells are involved.[2] When levels of gram-negative bacteria in the intestines are reduced by administration of antibiotics[3] or lactobacillus,[4] early alcohol-induced liver injury is also minimized, implicating endotoxin. Moreover, ethanol increases the permeability of the isolated small bowel to endotoxin in a dose-dependent manner.[5] Therefore, elevated circulating levels of endotoxin caused by excessive intake of alcohol could activate Kupffer cells to release many potent effectors and cytokines,[6] thus leading to alcohol-induced liver injury.[7] Tumor necrosis factor (TNF)-α levels are greater in alcoholics with hepatitis, and levels correlate with survival.[8] Because TNF-α is produced predominantly by the monocyte-macrophage lineage and the major population of this lineage in the liver is Kupffer cells,[9] increased production of TNF-α by activated Kupffer cells may be responsible for alcoholic hepatitis. Indeed, studies using the continuous intragastric feeding model in the rat of Tsukamoto and French[10,11] have shown that antibodies to TNF-α attenuate alcohol-induced liver injury.[12]

One approach to obtain clear evidence for or against the hypothesis of involvement of TNF-α in alcoholic liver disease is to use knockout mice. The advantage of using knockouts is that chemicals or antibodies that could have nonspecific effects are avoided. In addition, correlational analysis is not necessary for interpretation. Because gene-targeting technology has not been developed as extensively in rats as in mice, the surgical technique for enteral feeding was adapted to mice. TNF receptor 1 (TNF-R1) knockout mice were used to test the hypothesis that TNF-α participates in the pathogenesis of alcohol-induced liver injury. Zhang-Gouillon et al.[13] delivered alcohol enterally to mice in their studies of Mallory bodies in drug-primed mice, showing that successful surgery in the mouse was possible; however, the rate of mortality was high, possibly as a result of drug treatment.

Abbreviations used in this paper: dsDNA, double-stranded DNA; IFN, interferon; TGF, transforming growth factor; TNF-α, tumor necrosis factor α; TNF-R1, tumor necrosis factor receptor 1; TNF-R2, tumor necrosis factor receptor 2.

0016-5085/99/$10.00

The multiple biological activities of TNF-α are mediated by 2 distinct cell surface receptors of 55 kilodaltons (receptor 1) and 75 kilodaltons (receptor 2). Pfeffer et al.[14] generated a mouse strain (C57BL/6 background) lacking TNF receptor p55. It was observed that the TNF-R1 knockout rendered mice resistant to lethal doses of endotoxin,[14] indicating that TNF-R1 plays an important role in endotoxin-mediated processes. The present study is the first to successfully combine the use of gene-knockout mice with long-term enteral alcohol feeding. After 4 weeks of continuous enteral ethanol feeding, not only is steatosis observed, but inflammation and pericentral necrosis also occur in livers of wild-type and TNF-R2 knockout mice. However, in mice lacking the TNF-R1, hepatic pathology due to alcohol was blocked. Therefore, it is concluded that TNF-α plays a critical role in mechanisms of early alcohol-induced liver injury.

Materials and Methods

Animals

A breeding colony of TNF-R1 knockout mice (p55 −/−) was established at the University of North Carolina at Chapel Hill from breeding pairs kindly donated by Dr. Tak Mak of Amgen Institute (Toronto, Ontario, Canada). Wild-type C57Bl/6J mice and TNF-R2 knockout mice (p75 −/−) were purchased from the Jackson Laboratory (Bar Harbor, ME). Experiments were performed with adult male mice weighing 22–26 g housed in a facility approved by the American Association for Accreditation of Laboratory Animal Care. All animals received humane care in compliance with institutional guidelines. Body weight was measured before surgery and at necropsy after 4 weeks of continuous delivery of control or ethanol-containing diet.

Surgery

Mice were fasted for 24 hours before surgery. The principal surgical procedures were similar to methods previously described by Tsukamoto et al.[10] and French et al. in rats,[11] with modifications based on the size of mice. Briefly, mice were anesthetized by inhalation of methoxyflurane (Mallinckrodt Veterinary, Inc., Mundelein, IL), and laparotomy was performed under sterile surgical conditions. A 40-cm-long PE-90 polyethylene tube (Becton Dickinson, Sparks, MD) with an internal diameter of 0.86 mm was placed in the squamous part of the stomach. The tube was anchored to the stomach wall with Dacron polyester and fixed to the abdominal wall. It was then tunneled subcutaneously to the dorsal aspect of the neck, followed by closing of the abdominal wall with 7-0 prolene sutures. The tube was then pulled through a 250P polysulfone attachment mouse button (Instech Laboratories, Plymouth Meeting, PA) and spring coil. Unlike the separated button and spring coil used in rats, this special mouse button was connected to the spring coil. The button was fixed under the skin with its metal spring coil outside of the body to protect the tube (Figure 1). The feeding tube was attached to an infusion pump by means of a swivel, allowing complete mobility of the mouse within the metabolic cage. Animals were allowed to recover for 1 week with free access to chow diet and water before starting alcohol-containing or control liquid diets.

Diets

The basic liquid diet was prepared according to the method of Thompson and Reitz as described previously[15,16] and supplemented with lipotropes as described by Morimoto et al.[17] The control diet (1.3 kcal/mL) contained corn oil as fat (34% of total calories), protein (23%), and carbohydrate (43%), plus minerals and vitamins. For the ethanol diet, dextrin-maltose was replaced isocalorically with ethanol. The liquid diet was fed continuously at the rate of 10–11 mL/day to achieve weight gain. For a mouse of 25 g, a delivery of 28 g · kg^{-1} · day^{-1} of ethanol could be achieved by feeding 10 mL of 7% ethanol diet per day (37% of total calories from ethanol). Throughout the experimental period of liquid diet delivery, mice had free access to cellulose pellets as a source of fiber (Harlan Teklad, Madison, WI).

Experimental Protocol

Wild-type, TNF-R1 knockout, and TNF-R2 knockout mice were randomly allocated into experimental groups and were fed either ethanol-containing or isocaloric control diet (n = 6–7 per group). High-fat liquid diets were infused continuously through an intragastric cannula for up to 4 weeks. The ethanol dose began with 16 g · kg^{-1} · day^{-1} and was

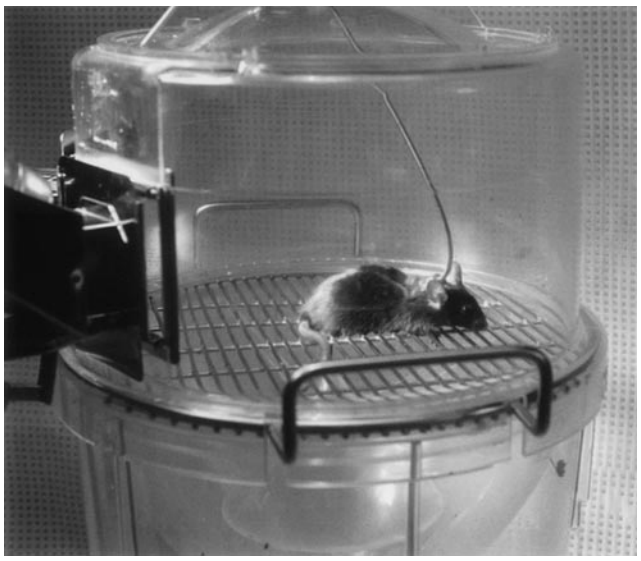

Figure 1. TNF-R1–deficient mouse after surgery. Detailed surgical procedures are described in Materials and Methods. The bottom end of the spring coil was fixed under the skin. The feeding tube outside of the body is protected by a spring coil and was connected to a feeding syringe driven by an infusion pump. Postoperatively, mice were kept in metabolic cages during the entire experimental period. Animals were allowed to recover from surgery for a week before the initiation of ethanol exposure and thereafter were observed carefully for signs of intoxication.

increased 1 $g \cdot kg^{-1} \cdot day^{-1}$ until the end of the first week (22 $g \cdot kg^{-1} \cdot day^{-1}$). This dose was maintained during the second week to allow the animals to establish tolerance to alcohol (see assessment system described below). From the beginning of the third week, the dose was increased 1 $g \cdot kg^{-1} \cdot day^{-1}$ until 28 $g \cdot kg^{-1} \cdot day^{-1}$ was achieved. This level of delivery was maintained until the end of the experiment. Animals receiving the ethanol diet were observed frequently for signs of alcohol intoxication. To achieve this goal, a scoring system of 0–3 was established: 0, normal behavior; 1, sluggish or staggering movement; 2, move with stimuli; 3, unresponsive or loss of consciousness. Tolerance to alcohol occurred during the second week of diet delivery as scores declined from 1 or 2 to 0. This method allowed administration of alcohol to be increased safely. After 4 weeks of liquid diet delivery with or without ethanol, mice were killed. Blood samples were collected via the inferior vena cava at necropsy, and serum was stored at −20°C until alanine aminotransferase (ALT) levels were analyzed by use of standard enzymatic procedures.[18] Livers were removed and weighed, and tissue samples were divided; some were fixed in formalin, and others were frozen in liquid nitrogen and stored at −80°C.

Urine Collection and Assay for Ethanol

Concentrations of ethanol in urine are representative of blood alcohol levels.[19] Mice were housed in metabolic cages that separated urine from feces, and urine samples were collected over 24 hours for each mouse. Ethanol levels in urine were determined daily by measuring the absorbance at 366 nm resulting from the reduction of nicotinamide adenine dinucleotide to the reduced form of nicotinamide adenine dinucleotide by alcohol dehydrogenase.[18]

Pathological Evaluation

Formalin-fixed liver samples were embedded in paraffin and stained with H&E to assess for steatosis, inflammation, and necrosis. Liver pathology was scored as described by Nanji et al.[20] as follows. Steatosis (the percentage of liver cells containing fat): <25%, 1+; <50%, 2+; <75%, 3+; >75%, 4+; inflammation and necrosis: 1 focus per low-power field, 1+; 2 or more, 2+. One point was given for each grade of severity of histological abnormality as described, and a total score was calculated for each liver.

Quantitation of Steatosis Using Image Analysis

A Universal Imaging Corp. Image-1/AT image-acquisition and analysis system (Universal Imaging Corp., Chester, PA) incorporating an Axioskop 50 microscope (Carl Zeiss, Inc., Thornwood, NY) was used to capture and analyze tissue sections at 200× magnification by using a modification of previously published techniques.[21] Color detection ranges were set for white areas representing fatty vacuoles. The extent of fat accumulation in the midzonal regions of the liver lobule was defined as the percentage of the field area within the default color range determined by the software, avoiding the influence of lumina of central veins. Average measurements from each tissue section (5 fields per section) were pooled to determine means.

Quantitation of Infiltrating Leukocytes and Necrosis

The total number of infiltrating leukocytes (including neutrophils and mononuclear cells), hepatocytes, and necrotic hepatocytes were counted in a 100-mm^2 area with a magnification of 200×. Five areas per section were randomly selected and counted, avoiding large lumina of vessels, and data were pooled to determine means.

Ribonuclease-Protection Assay of Cytokines

The double-stranded DNA (dsDNA) template for mouse cytokines (mCK3b) was purchased from Pharmingen (San Diego, CA). ^{32}P-labeled complementary RNA probes were produced from dsDNA templates by using the MAXIscript T7 kit from Ambion (Austin, TX) according to the manufacturer's instructions. Total RNA from liver samples was isolated by using the Qiagen RNeasy kit (Qiagen, Valencia, CA). Analysis of RNA by a ribonuclease (RNase)-protection assay was performed by using the Ambion RPA III Kit (Ambion, Austin, TX). Samples were electrophoresed on a sequencing gel, and protected fragments were quantitated by using a phosphor imaging system and ImageQuant software (Storm; Molecular Dynamics, Sunnyvale, CA). Messenger RNA (mRNA) levels of TNF-α, interferon (IFN)-γ, lymphotoxin β, transforming growth factor (TGF)-β, and macrophage migration inhibitory factor were quantitated.

Statistics

Analysis of variance (ANOVA) was used for the determination of statistical significance as appropriate. For comparison of pathological scores, the Mann–Whitney rank sum test was used. Data are presented as means ± SEM. $P < 0.05$ was selected before the study as the level of significance.

Results

Body Weights and Urine Levels of Ethanol

Figure 1 shows a typical adult male mouse from this study. All animals survived surgery and gained weight during the subsequent 4 weeks of continuous delivery of the liquid diets with or without ethanol (Table 1). Similar weight gains occurred in wild-type adult mice on chow diet (data not shown), and there were no significant differences in body weights between the groups studied. The reason for the relatively small weight gain observed was that the mice used in this study were adults; similar weight gains are observed in adult chow-fed C57Bl mice. Representative graphs of urine ethanol concentrations measured daily during 4 weeks of ethanol exposure are shown in Figure 2. As reported previously in

Table 1. Changes in Body Weight and Liver/Body Weight Ratio in Mice After 4 Weeks of Enteral Control or Ethanol-Containing Diet

Mice	Diets	Preoperative weight (*g*)	Weight at necropsy (*g*)	Liver/ body wt ratio (%)
Wild-type[a]	Control	26.5 ± 0.5	27.5 ± 0.2	5.5 ± 0.2
Wild-type	Ethanol	25.7 ± 0.3	26.8 ± 0.3	8.3 ± 0.8[d]
TNF-R1 KO[b]	Control	25.5 ± 0.5	26.0 ± 1.5	5.0 ± 0.7
TNF-R1 KO	Ethanol	24.4 ± 0.4	24.7 ± 0.5	6.7 ± 0.3
TNF-R2 KO[c]	Control	25.6 ± 0.3	26.8 ± 0.7	5.4 ± 0.1
TNF-R2 KO	Ethanol	26.0 ± 0.5	27.0 ± 0.6	8.8 ± 0.3[d]

[a]Wild-type, C57Bl/6J mice; [b]TNF-R1 KO, TNF-R1 knockout mice (p55 −/−); [c]TNF-R2 KO, TNF-R2 knockout mice (p75 −/−).
[d]$P < 0.05$ vs. mice of the same genetic background fed a control diet (1-way ANOVA).

studies with rats,[2,22] the urine levels of ethanol also fluctuated in the mouse in a cyclic pattern from 10 to >500 mg/dL, even though ethanol was infused at a continuous rate. Reasons for this phenomenon remain unknown, but at least it can be concluded that this phenomenon is not specific to the rat. Cyclic patterns of ethanol in the urine were similar in all groups studied. Average urine concentrations of ethanol during 4 weeks of ethanol exposure were 157 ± 25 mg/dL in wild-type mice, 170 ± 26 mg/dL in the TNF-R1 knockout mice, and 157 ± 23 mg/dL in TNF-R2 knockout mice, values that were not statistically different.

Liver/Body Weight Ratio

Liver/body weight ratios (Table 1) in wild-type mice receiving control diet (5.5% ± 0.2%) were significantly less than in wild-type mice fed ethanol (8.3% ± 0.8%, $P < 0.05$). However, the ratio in the TNF-R1 knockout mice given the control diet was not different from that in the TNF-R1 knockout mice receiving ethanol, indicating that ethanol caused greater enlargement of livers in wild-type than in TNF-R1 knockout mice. The ratios in the TNF-R2 knockout mice with or without ethanol in their diet (8.8 ± 0.3 and 5.4 ± 0.1) were similar to those of wild-type mice fed ethanol or control diets.

Serum Transaminase Levels

Before continuous administration of liquid diets, the average blood level of ALT in both wild-type and TNF-R1 knockout mice was 30 ± 4 U/L. In wild-type mice, 4 weeks of ethanol exposure significantly increased the serum ALT concentration ~3-fold (91 ± 5 U/L) over control values (Figure 3). Moreover, ALT levels from ethanol-fed TNF-R2 knockout mice (69 ± 9 U/L) were more than 2-fold higher than values from mice fed the control diet (34 ± 3 U/L; data not shown). Four weeks of ethanol feeding did not increase ALT levels in TNF-R1 knockout mice, however.

Pathological Evaluation

Figure 4 shows representative photomicrographs of livers from wild-type and TNF-R1 knockout mice after 4 weeks of exposure to control or ethanol diets. There were no pathological changes in wild-type or TNF-R1 knockout mice receiving the control diet (Figure 4*A* and *B*). The pathological score of control wild-type and knockout livers was essentially zero (Figure 5). However, marked fatty accumulation and mild-to-moderate inflammation and necrosis were observed in wild-type mice given ethanol (Figure 4*C, E,* and *F*), with an average pathology score of 5.5 ± 0.6. This value was significantly higher than that in wild-type mice given the control diet (Figure 5). Similar results were found with

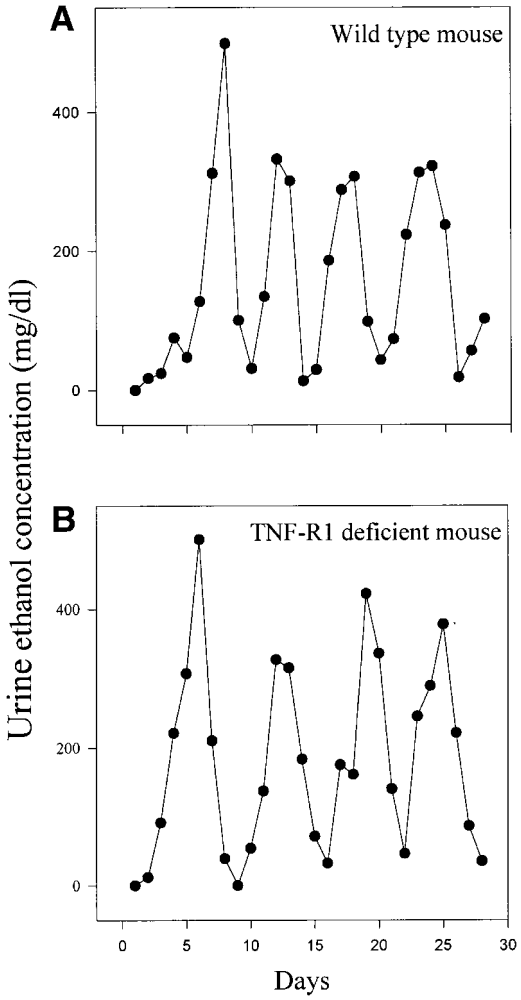

Figure 2. Representative plots of daily urine concentrations of alcohol in ethanol-fed mice. Urine was collected in metabolic cages, and alcohol concentrations were measured daily as described in Materials and Methods. Representative plots of urine ethanol concentration of a (*A*) wild-type and (*B*) TNF-R1–deficient mouse. Results of this experiment were typical.

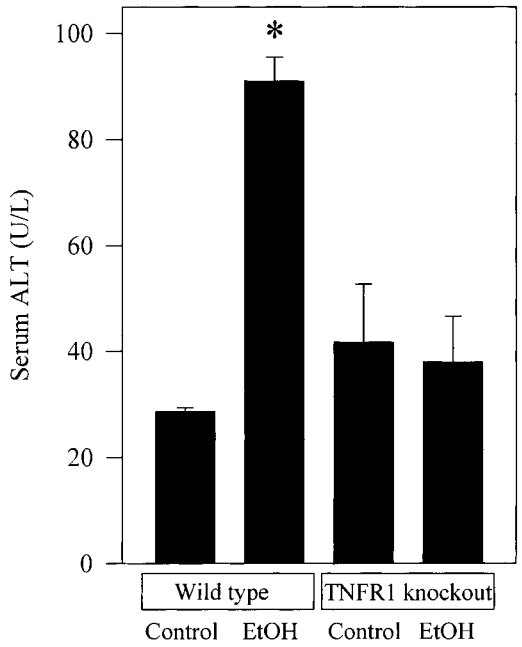

Figure 3. Effect of continuous diet delivery with or without ethanol on serum levels of ALT. Blood samples were collected at necropsy, i.e., 4 weeks after high-fat liquid diet feeding in the presence or absence of ethanol. ALT levels were determined as described in Materials and Methods. Data are means ± SEM. *$P < 0.05$ vs. other 3 groups of mice (2-way ANOVA).

TNF-R2 knockout mice. The pathology score was 0.3 ± 0.2 from TNF-R2 knockout mice fed a control diet, which was significantly smaller than that of ethanol-fed TNF-R2 knockout mice (5.0 ± 0.4). Fatty accumulation in wild-type mice receiving ethanol was panlobular but unevenly distributed (Figure 4*C*). Mild steatosis with small fat droplets was observed in the first 1–3 layers of hepatocytes around the central vein. This mild fatty accumulation was closely surrounded by very intensive steatosis, with massive macrodroplets of fat in the pericentral areas and midzonal regions near the central vein. The extent of steatosis from the middle of the midzonal to portal regions was moderate. In livers from TNF-R1–deficient mice, however, only very mild fatty accumulation was detected (Figure 4*D, G,* and *H*), with no coexisting inflammation or necrosis. As a result, the pathology scores of livers from TNF-R1 knockout mice receiving the ethanol diet were 0.8 ± 0.4 (Figure 5), values that were significantly lower than those in the ethanol-treated wild-type mice (5.5 ± 0.6) and the TNF-R2 knockout mice (5.0 ± 0.4; $P < 0.05$). In the present study, it was not possible to detect apoptosis because of severe fatty accumulation.

Image Analysis of Steatosis

The percentage of tissue area showing steatosis as determined by image analysis is shown in Figure 6. There was no fatty accumulation detected in livers from wild-type or TNF-R1 knockout mice fed the control diet. However, 4 weeks of ethanol exposure caused steatosis in nearly 13% of the midzonal area in livers from wild-type animals. TNF-R1 knockout mice, however, showed significantly less steatosis than wild-type mice (3.9%; $P < 0.001$).

Quantitation of Infiltrating Leukocytes and Necrosis

Infiltrating leukocytes (including neutrophils and mononuclear cells) and necrotic hepatocytes were observed in livers from ethanol-treated wild-type and TNF-R2 knockout mice. Leukocytes tended to be in foci, whereas necrotic cells were scattered. The number of leukocytes in liver before ethanol exposure was 0.7 per 100 hepatocytes, values that were not significantly different from those in livers of animals fed a control diet for 4 weeks (1/100 hepatocytes). Inflammation and necrosis in livers from TNF-R2 knockout mice fed ethanol showed a similar pattern. After 4 weeks of ethanol exposure, however, the total number of infiltrating leukocytes in wild-type mice increased ~7-fold (Figure 7*A*). In contrast, this infiltration was not observed in TNF-R1 knockout mice exposed to ethanol ($P < 0.05$). In addition, necrosis was barely detectable in wild-type or TNF-R1 knockout mice given the control diet. Four weeks of ethanol feeding increased necrosis in wild-type mice but not in TNF-R1 knockout mice (5.7 ± 0.2 vs. 1.6 ± 0.3; $P < 0.05$) (Figure 7*B*).

Cytokine Analysis

TNF-α mRNA levels were quantitated by use of an RNase-protection assay (Figure 8). Ethanol caused an ~2-fold increase in tissue levels of TNF-α mRNA in livers from wild-type and TNF-R1 knockout mice. There were no significant differences between these two ethanol-treated groups. However, 4 weeks of ethanol exposure did not result in alterations in mRNA levels for IFN-γ, lymphotoxin β, TGF-β, or macrophage migration inhibitory factor in livers from wild-type or TNF-R1 knockout mice (Table 2).

Discussion

Application of Continuous Enteral Alcohol Delivery to the Mouse

Typical histopathologic findings from livers of wild-type mice fed an ethanol-containing diet indicate that the long-term enteral feeding mouse model is practical. Although surgery in mice is somewhat more difficult than in rats because of their smaller body size,

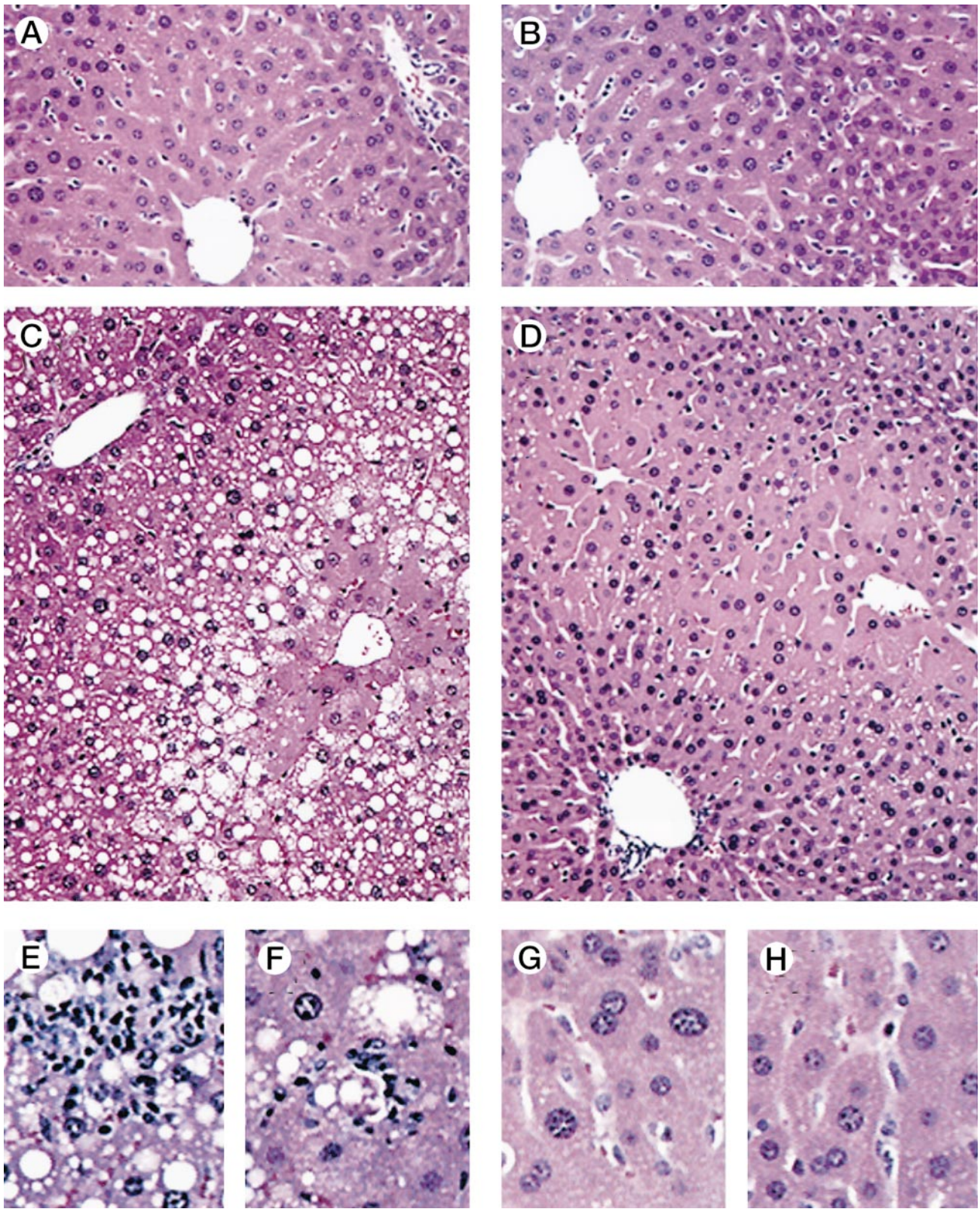

Figure 4. Representative photomicrographs of livers from wild-type and TNF-R1 knockout mice after 4 weeks of continuous diet delivery with or without ethanol. Animals were treated as described in Materials and Methods. Livers from (*A*) wild-type mice receiving a control diet, (*B*) TNF-R1 knockout mice fed a control diet, (*C*) wild-type mice given an ethanol diet, and (*D*) TNF-R1 knockout mice given an ethanol diet (original magnification 100×). With higher magnification, *E* and *F* show inflammation and necrosis in wild-type animals fed ethanol; *G* and *H* depict no inflammation or necrosis in TNF-R1–deficient mice fed ethanol.

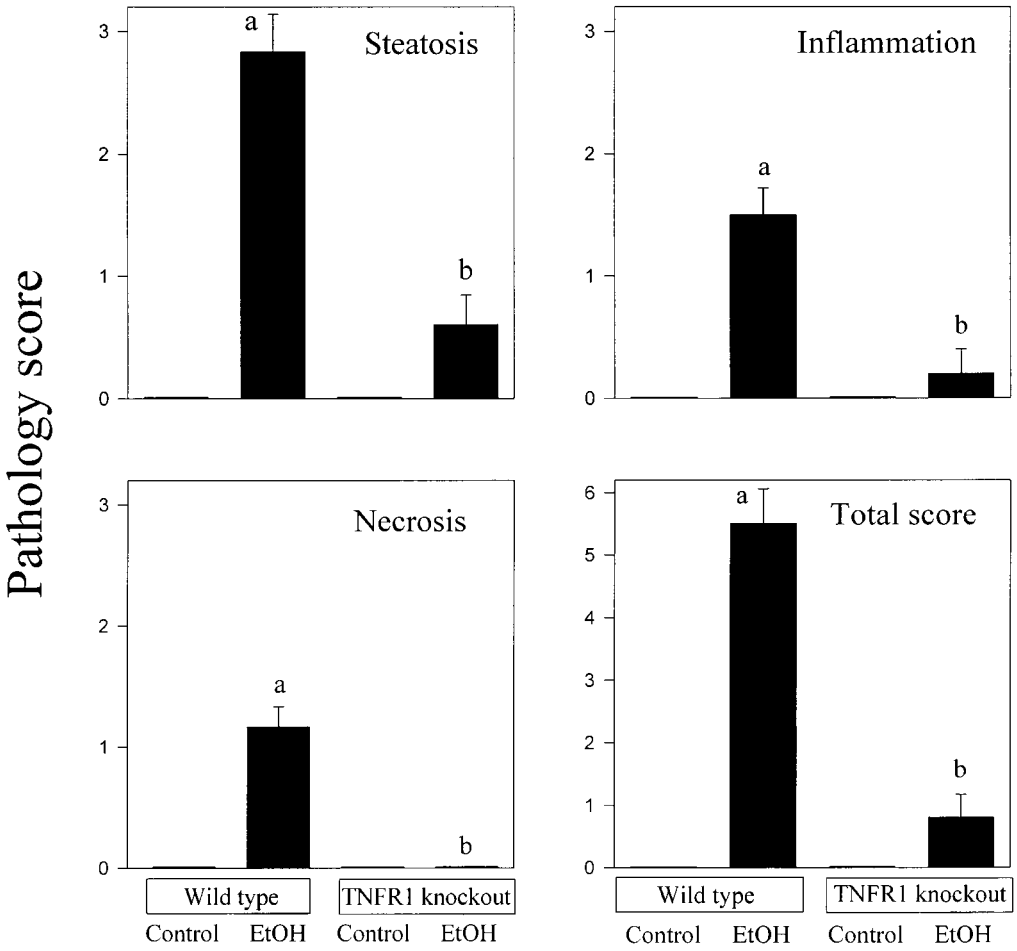

Figure 5. Effects of 4 weeks of continuous diet delivery with or without ethanol on hepatic pathology in wild-type and TNF-R1 knockout mice. Pathology was scored as described in Materials and Methods. EtOH, ethanol-containing diet. Data are means ± SEM. $^{a}P < 0.05$ vs. wild-type mice given control diet; $^{b}P < 0.05$ vs. wild-type mice given ethanol diet (Mann–Whitney rank sum test).

>90% postoperative survival rates can be achieved with care. For optimal survival rates, it was important that the animals be allowed to recover from surgery for 1 week before the liquid diets were initiated. During the recovery period, mice should have free access to chow and water. Throughout the continuous ethanol-feeding period, careful monitoring for alcohol toxicity was critical for optimal survival, because mice do not recover from it as well as rats. During the continuous ethanol-feeding period, mice were active and exhibited grooming behavior.

The role of carbohydrate in the pathogenesis of alcohol-induced liver injury is controversial.[23,24] In this study, liver pathology was markedly different between wild-type and TNF-R1 knockout mice fed the same ethanol diets, suggesting that dietary carbohydrate is not a key factor in the disease process, because the only difference between the 2 experimental groups was the presence or absence of TNF-R1.

Advantages of Use of Knockouts

Knockout technology, developed largely by Oliver Smithies and his group,[25] involves gene targeting of cultured embryonic stem cells, and homologous recombination allows insertion of DNA into the genome. Embryonic stem cells are injected into a fertilized egg and introduced into a host mother, and offspring are tested for mutations and characterized. The advantage of this technology is that specific deletions on disruption in DNA result in alterations that do not require the use of potentially nonspecific inhibitors or rely on correlations for interpretation of data. A specific genetic manipulation allows deletion of a key receptor or enzyme so that potentially nonspecific agents can be avoided, making interpretation of the data reliable. Moreover, the number of knockout mice that have become available in recent years has multiplied rapidly.

Role of TNF-α in Early Alcohol-Induced Liver Injury

TNF-α is a central proinflammatory cytokine,[26,27] and it has been suggested that it is important in the development of alcohol-induced liver injury.[12,28] However, until now our knowledge of TNF-α in the pathogenesis of alcoholic liver disease has come from in vitro

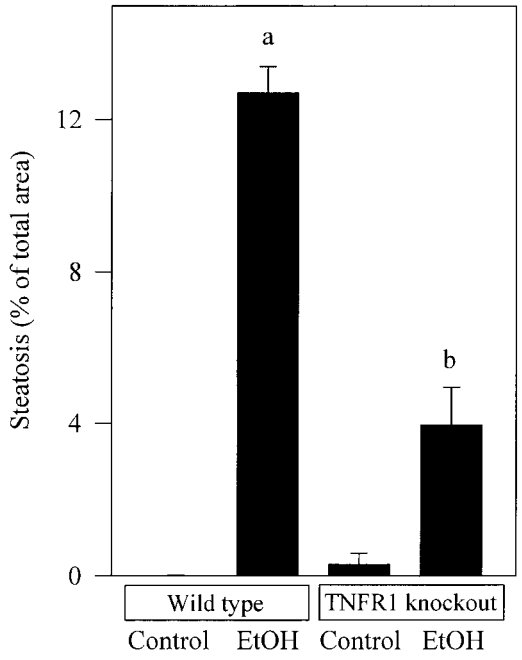

Figure 6. Effects of 4 weeks of continuous diet delivery with or without ethanol on hepatic steatosis in wild-type and TNF-R1 knockout mice. Animal treatments and image analysis of steatosis are described in Materials and Methods. EtOH, ethanol-containing diet. Data are means ± SEM. [a]$P < 0.01$ vs. all other groups of mice given control or ethanol diet; [b]$P < 0.01$ vs. TNF-R1–deficient mice given control diet (two-way ANOVA).

cultures of liver parenchymal and nonparenchymal cells,[28,29] in vivo measurement of TNF levels,[8,30–32] and in vivo neutralization with anti–TNF-α antibody.[12] Therefore, the use of animals lacking TNF receptors provides a direct way to test the hypothesis that this cytokine is important in initiation and progression of alcohol-induced liver injury.

The results of the present study show that TNF-α plays a major role in the pathogenesis of alcoholic liver injury, because ethanol-induced liver injury was minimized in mice lacking TNF-R1. After 4 weeks of continuous ethanol exposure, serum levels of ALT (Figure 3), liver pathology (Figures 4 and 5), hepatic steatosis (Figure 6), and inflammation and necrosis (Figure 7) were elevated only in mice that had TNF-R1 (i.e., in wild-type or TNF-R2 knockout mice). TNF-α activity has been shown to be mediated by 2 distinct receptors: receptor 1 (p55) and receptor 2 (p75).[33] These receptors are expressed in relatively different quantities on nearly all mammalian cells. In inflammation, the receptors activate both unique and synergistic responses.[34] Studies of TNF receptor knockout mice have established that p55 plays a predominant role in lipopolysaccharide-induced inflammatory diseases[35] and mediates the lethal effects of endotoxin. Furthermore, mice lacking TNF-R1 showed resistance to endotoxin challenge.[36] This idea is supported by the observation in the present study that pathology due to alcohol consumption was not blocked in wild-type or TNF-R2 knockout mice (see Results). It has been shown previously that early alcohol-induced liver injury was blocked by intestinal sterilization with antibiotics and lactobacillus to reduce levels of endotoxin,[3,4] the use of $GdCl_3$ to kill Kupffer cells,[2] and the administration of antibodies to TNF-α.[12] Therefore, the reduced liver pathology in the TNF-R1 knockout mice we observed is consistent with the hypothesis that excessive intake of alcohol increases circulating levels of endotoxin, which activates Kupffer cells to release TNF-α, leading to liver injury. TNF-α mRNA levels were increased by ethanol in the livers from both wild-type and TNF-R1 knockout mice, supporting the involvement of TNF-α.

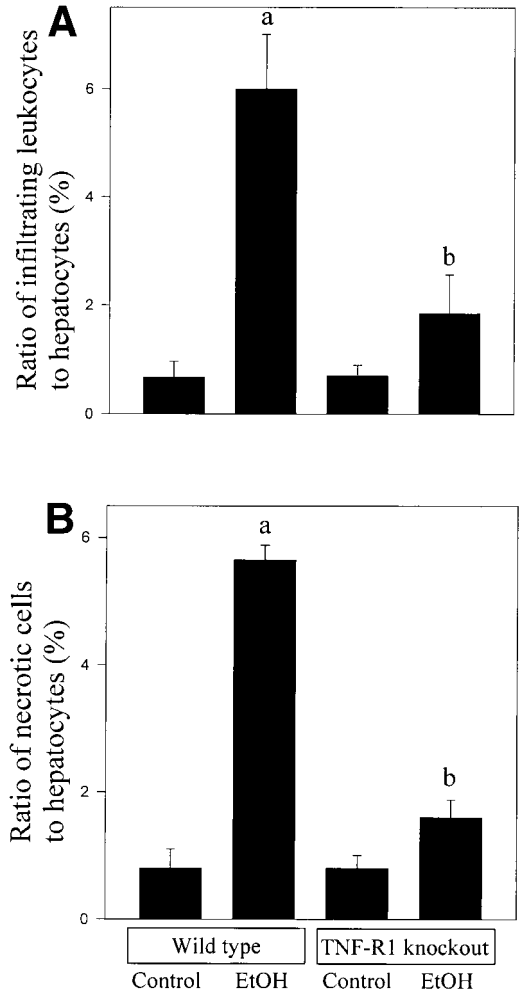

Figure 7. Effects of 4 weeks of continuous diet delivery with ethanol on hepatic (*A*) inflammation and (*B*) necrosis in wild-type and TNF-R1 knockout mice. Animal treatments and quantitation of infiltrating leukocytes and necrotic cells are described in Materials and Methods. EtOH, ethanol-containing diet. Data are means ± SEM. [a]$P < 0.01$ vs. all other groups of mice with control or ethanol diets; [b]$P < 0.05$ vs. the knockouts given control diet (two-way ANOVA).

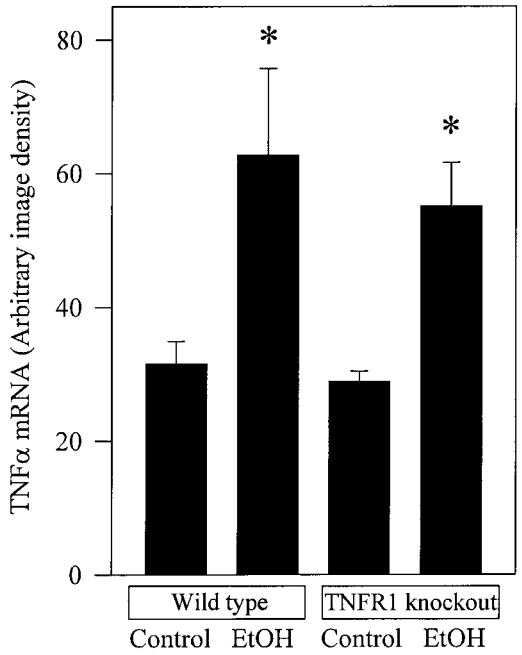

Figure 8. Effects of 4 weeks of continuous diet delivery with ethanol on tissue levels of TNF-α mRNA in livers from wild-type and TNF-R1 knockout mice. TNF-α mRNA levels were measured by RNase-protection assay as detailed in Materials and Methods. TNF-α mRNA levels are expressed relative to glyceraldehyde-3-phosphate dehydrogenase in arbitrary units. $*P < 0.05$ vs. wild-type or TNF-R1 knockout mice fed control diet (one-way ANOVA).

One of the main features of early ethanol-induced liver injury is steatosis, which was largely blocked in the TNF-R1–deficient mice (Figure 6). This reduced steatosis in the knockout mice indicates that TNF-α is involved. It is known that TNF-α released by endotoxin-activated Kupffer cells[37] stimulates lipid synthesis in the liver.[38,39] Feingold and Grunfeld[38] showed that the synthesis of fatty acids in liver was increased 1–2 hours after TNF-α administration, an effect that persisted for >17 hours. Total hepatic triglyceride production, measured by the incorporation of tritiated glycerol into hepatic serum triglycerides, was increased in TNF-α–treated animals.[40] TNF-α also stimulated peripheral lipolysis,[41] leading to an increase in circulating levels of free fatty acids, resulting in increased delivery of lipid to the liver, where it was reesterified.[42] A decrease in lipoprotein lipase activity by TNF-α is responsible for decreased clearance of triglyceride-rich lipoproteins, such as very low density lipoproteins, leading to hyperlipidemia.[43] Thus, it is concluded that reduced fat accumulation in livers of ethanol-treated mice lacking TNF-R1 is the result of the loss of effects of TNF-α on lipid synthesis and mobilization. This phenomenon is TNF-R1 specific, because it did not occur in TNF-R2 knockout mice. It is also possible that other mechanisms involved in steatosis are compromised in the TNF-R1 knockout mice.

In general, steatosis was observed in all regions of the liver lobule in livers from wild-type mice fed an ethanol diet; however, the most severe fatty accumulation was found predominantly in pericentral areas, except for 1–3 layers of hepatocytes that immediately surround the central vein. Reasons for this phenomenon are not clear; however, Arteel et al. showed that these cells were anoxic in rats.[44] Thus, decreased lipid accumulation in the last cell layers may be caused by a lack of adenosine triphosphate for the synthesis of triglycerides in the most oxygen-poor pericentral regions. However, this zonation was not observed in previous studies with rats but was detected in other experiments with mice,[45] leading to the conclusion that it is mouse specific.

In summary, TNF-α plays a major role in vivo in the pathogenesis of alcohol-induced liver injury; thus, drugs that target TNF-α signaling pathways via the receptor-1 pathway may prove beneficial in treating alcoholic hepatitis. The present study shows a stable and practical model of long-term enteral ethanol feeding in the mouse. This technique, combined with transgenic technology, may provide extremely powerful tools for elucidating the complex mechanisms involved in early alcohol-induced liver disease.

Table 2. Cytokine Levels in Livers From Mice After 4 Weeks of Enteral Control or Ethanol-Containing Diet

Mice	Diets	IFN-γ	LT-β	TGF-β	MIF
Wild-type	Control	46 ± 4	14 ± 1	13 ± 2	46 ± 3
Wild-type	Ethanol	74 ± 12	17 ± 2	17 ± 1	57 ± 3
TNF-R1 KO	Control	40 ± 4	12 ± 1	13 ± 1	59 ± 4
TNF-R1 KO	Ethanol	66 ± 7	17 ± 1	16 ± 1	64 ± 3

NOTE. Cytokine levels in livers were determined by use of an RNase-protection assay as described in Materials and Methods. Statistical differences between means were determined by using one-way ANOVA.

Wild-type, wild-type C57Bl/6J mice; TNF-R1 KO, TNF-R1 knockout mice (p55 −/−); LT-β, lymphotoxin β; MIF, macrophage migration inhibitory factor.

References

1. Felver ME, Mezey E, McGuire M, Mitchell MC, Herlong HF, Veech GA, Veech RL. Plasma tumor necrosis factor α predicts decreased long term survival in severe alcoholic hepatitis. Alcohol Clin Exp Res 1990;14:255–259.
2. Adachi Y, Bradford BU, Gao W, Bojes HK, Thurman RG. Inactivation of Kupffer cells prevents early alcohol-induced liver injury. Hepatology 1994;20:453–460.
3. Adachi Y, Moore LE, Bradford BU, Gao W, Thurman RG. Antibiotics prevent liver injury in rats following long-term exposure to ethanol. Gastroenterology 1995;108:218–224.
4. Nanji AA, Khettry U, Sadrzadeh SMH. *Lactobacillus* feeding reduces endotoxemia and severity of experimental alcoholic liver disease. Proc Soc Exp Biol Med 1994;205:243–247.

5. Arai M. Effect of ethanol on the intestinal uptake of endotoxin (abstr). Nippon Shokakibyo Gakkai Zasshi 1986;83:1060.
6. Decker K. Biologically active products of stimulated liver macrophages (Kupffer cells). Eur J Biochem 1990;192:245–261.
7. Thurman RG. Mechanisms of hepatic toxicity. II. Alcoholic liver injury involves activation of Kupffer cells by endotoxin. Am J Physiol 1998;275:G605–G611.
8. Stahnke LL, Hill DB, Allen JI. TNFα and IL-6 in alcoholic liver disease. In: Wisse E, Knook DL, McCuskey RS, eds. Cells of the hepatic sinusoid. 3rd ed. Leiden, The Netherlands: Kupffer Cell Foundation, 1991:472–475.
9. Decker T, Lohmann-Matthes ML, Karck U, Peters T, Decker K. Comparative study of cytotoxicity, tumor necrosis factor, and prostaglandin release after stimulation of rat Kupffer cells, murine Kupffer cells, and murine inflammatory liver macrophages. J Leukoc Biol 1989;45:139–146.
10. Tsukamoto H, Reiderberger RD, French SW, Largman C. Long-term cannulation model for blood sampling and intragastric infusion in the rat. Am J Physiol 1984;247:R595–R599.
11. French SW, Miyamoto K, Tsukamoto H. Ethanol-induced hepatic fibrosis in the rat: role of the amount of dietary fat. Alcohol Clin Exp Res 1986;10:13S–19S.
12. Iimuro Y, Gallucci RM, Luster MI, Kono H, Thurman RG. Antibodies to tumor necrosis factor-α attenuate hepatic necrosis and inflammation due to chronic exposure to ethanol in the rat. Hepatology 1997;26:1530–1537.
13. Zhang-Gouillon ZQ, Yuan Q-X, Hu B, Marceau N, French BA, Gaal K, Nagao Y, Wan Y-JY, French SW. Mallory body formation by ethanol feeding in drug-primed mice. Hepatology 1998;27:116–122.
14. Pfeffer K, Matsuyama T, Kundig TM, Wakeham A, Kishihara K, Shahinian A, Wiegmann K, Ohashi PS, Kronke M, Mak TW. Mice deficient for the 55 kD tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to *L. monocytogenes* infection. Cell 1993;73:457–467.
15. Thompson JA, Reitz RC. Effects of ethanol ingestion and dietary fat levels on mitochondrial lipids in male and female rats. Lipid 1978;13:540–550.
16. Iimuro Y, Bradford BU, Forman DT, Thurman RG. Glycine prevents alcohol-induced liver injury by decreasing alcohol in the stomach. Gastroenterology 1996;110:1536–1542.
17. Morimoto M, Zern MA, Hagbjork AL, Ingelman-Sundberg M, French SW. Fish oil, alcohol, and liver pathology: role of cytochrome P4502E1. Proc Soc Exp Biol Med 1994;207:197–205.
18. Bergmeyer HU. Methods of enzymatic analysis. New York: Academic, 1988.
19. Badger TM, Crouch J, Irby D, Shahare M. Episodic excretion of ethanol during chronic intragastric ethanol infusion in the male rat: continuous vs. cyclic ethanol and nutrient infusions. J Pharmacol Exp Ther 1993;264:938–943.
20. Nanji AA, Mendenhall CL, French SW. Beef fat prevents alcoholic liver disease in the rat. Alcohol Clin Exp Res 1989;13:15–19.
21. Arteel GE, Raleigh JA, Bradford BU, Thurman RG. Acute alcohol produces hypoxia directly in rat liver in vivo: role of Kupffer cells. Am J Physiol 1996;271:G494–G500.
22. Iimuro Y, Frankenberg MV, Arteel GE, Bradford BU, Wall CA, Thurman RG. Female rats exhibit greater susceptibility to early alcohol-induced injury than males. Am J Physiol 1997;272:G1186–G1194.
23. Badger TM, Korourian S, Hakkak R, Ronis MJJ, Shelnutt SR, Ingelman-Sundberg M, Waldron I. Carbohydrate deficiency as a possible factor in ethanol-induced hepatic necrosis. Alcohol Clin Exp Res 1998;22:742.
24. French SW, Zhang-Gouillon ZQ, Ingelman-Sundberg M. The role of CYP2E1 induction by ethanol in the pathogenesis of alcoholic liver disease as determined by inhibitors of CYP2E1 transcription and posttranslation modulating factors. Alcohol Clin Exp Res 1998;22:738–739.
25. Smithies O. Animal models of human genetic diseases. Trends Genet 1993;9:112–116.
26. Strieter RM, Kunkel SL, Bone RC. Role of tumor necrosis factor–alpha in disease states and inflammation. Crit Care Med 1993;21:S447–S463.
27. Probert L, Akassoglou K, Alexopoulou L, Douni E, Haralambous S, Hill S, Kassiotis G, Komtoyiannis D, Pasparakis M, Plows D, Kollias G. Dissection of the pathologies induced by transmembrane and wild-type tumor necrosis factor in transgenic mice. J Leukoc Biol 1996;59:518–525.
28. Deaciuc IV, D'Souza NB, Spitzer JJ. Tumor necrosis factor–alpha cell-surface receptors of liver parenchymal and nonparenchymal cells during acute and chronic alcohol administration to rats. Alcohol Clin Exp Res 1995;19:332–338.
29. Hill DB, McClain CJ, St Clair D, Barve S. Use of transfected liver cells to evaluate potential mechanism of alcohol-induced liver injury. Alcohol Clin Exp Res 1998;22:785–788.
30. Nanji AA, Zhao S, Sadrzadeh SM, Waxman DJ. Use of reverse transcription–polymerase chain reaction to evaluate in vivo cytokine gene expression in rats fed ethanol for a long period. Hepatology 1994;19:1483–1487.
31. Yin M, Ikejima K, Arteel GE, Seabra V, Bradford BU, Kono H, Roussyn I, Thurman RG. Glycine accelerates recovery from alcohol-induced liver injury. J Pharmacol Exp Ther 1998;286:1014–1019.
32. Hansen J, Cherwitz DL, Allen JI. The role of tumor necrosis factor-alpha in acute endotoxin-induced hepatotoxicity in ethanol-fed rats. Hepatology 1998;20:461–474.
33. Peschon JJ, Torrance DS, Stocking KL, Glaccum MB, Otten C, Willis CR, Charrier K, Morrissey PJ, Ware CB, Mohler KM. TNF receptor–deficient mice reveal divergent roles for p55 and p75 in several models of inflammation. J Immunol 1998;160:943–952.
34. Tartaglia LA, Weber RF, Figari IS, Reynolds C, Palladino MA Jr, Goeddel DV. The two different receptors for tumor necrosis factor mediate distinct cellular responses. Proc Natl Acad Sci USA 1991;88:9292–9296.
35. Amar S, Van Dyke TE, Eugster HP, Schultze N, Keobel P, Bluethmann H. Tumor necrosis factor (TNF)-induced cutaneous necrosis is mediated by TNF receptor 1. J Inflamm 1995;47:180–189.
36. Acton RD, Dahlberg PS, Uknis ME, Klaerner HG, Fink GS, Norman JG, Dunn DL. Differential sensitivity to *Escherichia coli* infection in mice lacking tumor necrosis factor p55 or interleukin-1 p80 receptors. Arch Surg 1996;131:1216–1221.
37. Martinez F, Abril ER, Earnest DL, Watson RR. Ethanol and cytokine secretion. Alcohol 1992;9:455–458.
38. Feingold KR, Grunfeld C. Tumor necrosis factor–alpha stimulates hepatic lipogenesis in the rat *in vivo*. J Clin Invest 1987;80:184–190.
39. Feingold KR, Hardardottir I, Grunfeld C. Cytokine-induced alterations in hepatic lipid metabolism. In: Gerok W, Decker K, Andus T, Gross V, eds. Cytokines and the liver. Dordrecht, Netherlands: Kluwer Academic Publishers, 1994:172–181.
40. Feingold KR, Serio MK, Adi S, Moser AH, Grunfeld C. Tumor necrosis factor stimulates hepatic lipid synthesis and secretion. Endocrinology 1989;124:2336–2342.
41. Feingold KR, Doerrler W, Dinarello CA, Fiers W, Grunfeld C. Stimulation of lipolysis in cultured fat cells by tumor necrosis factor, interleukin-1, and the interferons is blocked by inhibition of prostaglandin synthesis. Endocrinology 1992;130:10–16.

42. Wolfe RR, Shaw JHF, Durkot MJ. Effect of sepsis on VLDL kinetics: responses in basal state and during glucose infusion. Am J Physiol 1985;248:E732–E740.
43. Kawakami M, Pekala PH, Lane MD, Cerami A. Lipoprotein lipase suppression in 3T3-L1 cells by an endotoxin-induced mediator from exudate cells. Proc Natl Acad Sci USA 1982;912–916.
44. Arteel GE, Iimuro Y, Yin M, Raleigh JA, Thurman RG. Chronic enteral ethanol treatment causes hypoxia in rat liver tissue *in vivo*. Hepatology 1997;25:920–926.
45. Kono H, Yin M, Bradford BU, Gonzalez FJ, Thurman RG. Development of a new enteral mouse model using knockout technology to study alcohol-induced liver injury: is CYP2E1 involved? (abstr). Am J Physiol 1999 (in press).

Received December 15, 1998. Accepted July 1, 1999.

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Supported in part by grants from the National Institute on Alcohol Abuse and Alcoholism.